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Characterization of bacterial strains associated with sheath rot complex and grain discoloration of rice in North of Iran (Mazandaran province)

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In recent years, similar symptoms to sheath rot complex (SH C) and grain discoloration (GD) has been observed in paddy fields in the north of Iran. To survey the etiology of this complex disease, 207 samples of rice plants showing wide range of sheath and grain discoloration were collected during 2002-2005 at booting and ripening stages from various geographical locations in Mazandaran province. Pathogenic strains were often isolated from samples with the symptom of longitudinal brown to reddish brown necrosis 5 mm wide extending the entire length of flag leaf sheath. Over 800 strains were isolated from collected samples and tested for pathogenicity on rice and/or hypersensitivity on tobacco or pelargonium. Pathogenic strains were isolated from 20.28% samples which contained only 5.3%of total strains. Eighty two strains comprising 72 strains from plants showing symptoms of sheath rot and grain discoloration, five strains from diseased seedling and five standard strains were analyzed for phenotypic studies. Pseudomonas-specific primers were used to confirm identification of the genus.On the basis of phenotypic characters and genus specific primers, the strains belonged to genera *Acidovorax* and *Pseudomonas*. Cluster analysis of 67 biochemical characters grouped 70 selected strains into seven distinct cluster and six groups with one member. The results confirmed that pathogenic strains associated with SH C and GD in Iran (Mazandaran province) belong to *Acidovorax avenae* subsp *avenae* (2.32%), *Pseudomonas putida* (2.32%), *Pseudomonas marginalis* (6.65%), *Pseudomonas syringae* (76.7%) and two unidentified species of *Pseudomonas* (13.95%). Accordingly, the *P. syringae* was revealed that is the major causal agent of SH C and GD in north of Iran.

Key words: *Oryza sativa* L., bacterial flag leaf sheath rot, *Pseudomonas*.

INTRODUCTION

Sheath rot complex and grain discoloration of rice (*Oryza sativa* L.) generally describes a disease which involves brown discoloration or the flag leaf sheath rot and grain discoloration. Both fungi and bacteria are reported to be associated with this disease (Cottyn et al., 1996a, b; Zeigler and Alvarez, 1990). The symptoms apparently caused by these agents are similar, preventing reliable diagnosis only based on symptomology (Duveiller et al., 1998; Cottyn et al., 1996a; Zeigler and Alvarez, 1990). This disease is widespread in temperate (Joana et al., 2007) and tropical areas (Cottyn et al., 1996a; Jaunet et al., 1996) and more prevalent in areas where low temperature (and humidity) occur during the rice booting and heading stages (Jaunet et al., 1996) and the disease is especially apparent during the rainy season and the intensity of infection varies from mild to severe (Cottyni et

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al.,1996). Several pathogenic bacteria have been associated to be the cause of this disease in various parts of the world such as Asia, Africa, Latin America, Europe and Australia (Cottyn et al., 1996a; Cottyn et al., 1996b; Rott et al., 1989; Zeigler and Alvarez, 1990; Zeigler et al., 1987; Xie et al., 2012; Adoradaet al., 2013). Bacterial sheath rot of rice caused by *Pseudomonas oryzicola* was first described in 1955 (Ou, 1985) but the name was changed later to *P. syringae pv. syringae* (Ou, 1985; Zeigler et al., 1987). *Pseudomonas fuscovaginae* which causes sheath brown rot was first reported by Tani et al. (Miyajima et al., 1983) in Hokkaido in the north of Japan. Subsequently, this pathogen was reported in Burundi, Colombia, Madagascar, China, Nepal and Philippines (Cottynet al., 1996 a, b; Duveiller et al., 1998; Xie, 2003; Zeigler and Alvarez, 1987). *Burkholderiaglumae* (the causal agent of both seedling and grain rot) and *Acidovorax avenae subsp avenae* (A causal agent of bacterial stripe) have been reported to be associated with this disease (Cottyn et al., 1996a; Zeigler and Alvarez, 1990). In addition to these bacterial pathogens that commonly reported to be involved in sheath rot complex and grain discoloration, bacteria with similar characteristics to *Pseudomonas marginalis*, *Pseudomonas fluorescens*, *Pseudomonas corruga*ta, *Pseudomonas aurofacience*, *P. fluorescens*bv. V and *P. fluorescens* bv. IV have been pathogenic and were able to induce sheath rot of rice (Joana et al., 2007; Cottyn et al., 1996). In Iran, a few surveys were made in paddy fields of Mazandaran province (the major rice growing region) to determine the causal agents of this complex disease. In one study, some fungi including *Sarocladium oryzae*, *Cochiobolus miyabeanus*, *Althernaria padwichi*i and *Fusarium* spp were confirmed to be associated with this disease (Naeimi et al., 1983)**.** There are two short reports on engagement of *Pseudomonas fuscovaginae* (Rostami et al., 1985) and *P. syringae* (Khoshkadam et al., 2008) in this disease .The present study was designed to investigate the occurrence of pathogenic bacteria involved in the sheath rot complex and grain discoloration in Iran (Mazandaran province).

MATERIALS AND METHODS

Isolation of bacteria from plant material

Diseased flag leaf sheath and grains of rice at booting and ripening stages were collected though out the geographical regions of Mazandaran province during 2002 - 2005. Small segments of sheath from the margin of discoloration tissue were washed under running tap water for 20 min and then macerated in sterile distilled water. Loopful of macerated tissue were streaked onto King's B medium and incubated at 28°C for 1 to 3 days. Three to five predominant colony types and all fluorescent colonies were purified by restreaking and kept on nutrient agar slants at 4°C for routine work. To isolate the pathogenic bacteria from seeds, about 20 seeds of each sample were washed under tap water for 30 min and the rest of the procedure was as described for sheath samples. All obtained strains were evaluated on rice at both seedling and booting stages and pathogenic strains at these stage as well as some non-pathogenic fluorescent strains and yellow pigment strains were selected for further studies.

Bacterial strains

A total number of 82 strains comprising72 strains from rice plants showing symptoms of sheath rot and grain discoloration, five strains from diseased seedling and five standard strains obtained from BSBF were studied (Table 1).

Pathogenicity test

Inoculations were made on rice Fajr and Tarom cultivars on both seedling and adult plants. Pathogencity teston seedling was conducted on 20 day old rice. Inoculation was made by injection of a suspension of 10^8cfu/ml of 24 h old bacterial culture in sterile water between leaf sheath located about 5 cm above soil (Duveiller et al., 1998; Rott et al., 1991). At least four seedlings were inoculated and for control two seedlings were injectedby sterile distilled water. The plants were incubated in a moist chamber at 100% relative humidity (RH) at 24°C for 48 h before being moved to greenhouse. The same type of inoculation was performed on the flag leaf sheath on adult plants at booting stage. The inoculated plants were incubated for 48 h at 100% RH at 24°C and transferred to the greenhouse (Duveiller et al., 1998; Zeigler and Alvarez, 1987; Zeigler and Alvarez, 1990). Two plants each with 5 tillers were inoculated and for control, two tillers were injected by sterile distilled water. The plants were evaluated in two day intervals during the first week and then in one- week intervals up to one month.

Biochemical characteristics

The physiological and morphological characteristics of 70 strains (Table 2) were determined by 67phenotypic tests using standard microbiological techniques (Schaad et al., 2001; Mew and Cottyn, 2001).

Genus-specific primers

Genus-specific 16Sr RNA of *Pseudomonas* (sensostrictu) were used for selective detection of strains of *Pseudomonas* (Widmer et al., 1992; Porteouset al., 1985).

DNA extraction

For genomic DNA preparation, the bacteria were grown on Tryptic soy broth (Merck), shaking for 24 - 48 h at 28°C. Cells were harvested by centrifugation (10/000 rpm, 5 min), washed with sterile water and resuspended in sterile distilled water to give an OD 600 of 0.5. Cells were lyzed by NaOH 3% (g/vol) and centrifuged (10/000 rpm, 5 min) to pellet cell debris. Clear supernatant DNA solution was extracted twice with chloroform. Precipitation of nucleic acids was made with sodium acetate (final concentration, 0.3 M) and 2 volume of absolute ethanol (Rahimian, unpublished). The DNA was dissolved in sterile water and the concentration of DNA was determined spectrophotometrically at 260 nm.

PCR amplification

The primers PS - for (20-mer[5'-GGTCTGAGAGGATGATCAGT-3']) and PS - rev (18 -mer [5'-TTAGCTCCACCTCGCGGC-3']) were used for PS – PCR (Widmer et al., 1992). Amplification reactions were performed in 20 μl of a solution containing 1X reaction buffer (

Table 1. Geographical origin, tissue of origin and pathogenicity test on rice forbacterial strains used in this study.

Location	Tissue origin	Rice pathogenicity		Strain number
		Seedling stage	Booting stage	
Amol	Sheath	+	+	1, 2, 18, 19, 43
	Sheath	W	+	3, 34
	Sheath	-	+	101
	Sheath	+	W	31
	Sheath	-	-	102, 103, 104, 105
	Sheath	ND	-	302, 303
	Seed	+	+	16, 17, 109
	Seed	-	-	108, 122
	Seedling	+	W	201
	Seedling	-	-	202
Babol	Sheath	+	+	4, 5, 20, 21
	Sheath	+	-	33
	Seed	-	-	110
	Seedling	-	-	203
Babolsar	Sheath	+	+	9, 10
	Seed	-	-	111
Chalos and Noshah	Sheath	W	+	42
	Sheath	W	-	36
	Seed	+	+	29
Ghaemshah	Sheath	+	+	11, 22, 23, 26
	Sheath	+	-	35
	Seed	+	+	8
	Seed	ND	-	304
Fridonkenar	Sheath	+	+	28
	Seed	-	-	120
	Seedling	+	W	204
Mahmodabad	Sheath	+	+	12, 13. 25, 30
	Sheath	+	-	39, 41
	Seed	+	+	27, 44
	Seed	W	W	32
	Seed	-	-	118
	Seedling	-	-	205
Noor	Sheath	+	+	14
	Sheath	W	W	37
	Seed	+	-	38
	Seed	-	-	40
Sari	Sheath	-	+	106
	Seed	-	+	112
	Seed	-	-	113, 121
Savadkouh	Sheath	-	W	107
	Sheath	+	-	24
	Seed	+	+	6, 7
	Seed	-	W	116, 117
Shiraze	Seed	-	-	123
Tonokabon	Seed	+	+	15
	Seed	-	-	115, 119
	Seed	-	w	114
NO	Seed	-	-	403
Strain standard				
IBSBF(674)	-	ND	ND	401(*P. viridiflava*Typestrine)
IBSBF(676)	-	ND	ND	402 (*P. s.* pv. *panici*)
IBSBF (674)	-	ND	ND	404 (*P. s.* pv. *atrofaciens*)
IBSBF (199)	-	ND	ND	405(*B.andropogonis*,Typestrine)
IBSBF (1890)	-	ND	ND	406 (*B. gladioli*pv. *gladiol*)

W, Weak pathogenesis; ND, not determined; NO, unknown.

Table 2. Phenotypic characteristics of strains associated with sheath rot complex and grain discoloration of rice in Mazandaran province (Iran).

Character [b]	Cluster number[a]												
	A	B	C	D	E	F	G	40	201	29	122	405	406
Gram stain(KoH)	-	-	-	-	-	-	-	-	-	-	-	-	-
Fluorescent pigment	+	+	+	+	+	+	+	+	+	-	+	-	-
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+
Levan production from sucrose	-	-	-	-	v	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	-	-	-	+	+	+	-	-	-
Soft rot of potato	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	+	+	+	+	-	-	-	+	+	+	-	+	+
Tobacco hypersensitivity	+	+	+	+	+	+	+	-	+	+	+	+	+
2 -ketogluconate production	+	v	v	+	-	+	v	+	-	-	-	-	-
Nitrate reduction	v	v	v	-	-	-	-	+	-	+	-	-	+
eculin hydrolysis	-	-	-	-	+	+	+	-	-	-	+	-	+
Arbutin hydrolysis	-	-	-	-	+	+	v	-	-	-	+	-	+
Starch hydrolysis	-	-	+	+	-	-	+	-	-	-	-	-	-
Gelatin liquefaction	-	+	+	+	v	-	+	+	+	-	-	-	-
Tween 80 hydrolysis	-	+	+	+	+	+	+	+	+	+	+	-	-
Casein hydrolysis	v	+	+	-	+	v	+	+	-	-	+	-	+
Urease production	+	+	+	+	+	+	+	+	-	+	+	+	-
Indol Production	-	-	-	-	-	-	-	-	-	-	-	-	-
Phosphatase	v	v	-	+	-	v	+	+	-	+	-	+	+
Production of reducing substance from sucrose	-	-	v	-	+	v	v	-	-	-	-	-	-
H2s from cysteine	-	-	v	v	-	-	v	+	-	+	+	-	-
H2s from thiosulfate	-	-	-	+	-	-	v	-	-	+	+	-	-
Growth in NaCl 3%	+	+	+	+	+	+	+	+	+	+	-	-	+
Growth in NaCl 5%	+	+	-	v	v	v	-	+	-	-	-	-	-
Fermentation Test	-	-	-	-	-	-	-	-	-	-	-	-	-
Lecithinase test	v	+	v	+	-	v	v	+	-	-	+	-	+
Voges-proskauer test	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red reaction	-	-	-	-	-	-	-	-	-	-	-	-	-
Ice nucleation	-	v	-	-	+	+	+	-	-	-	-	-	-
Growth at 37°C	+	+	v	v	-	+	-	+	+	+	+	-	+
Growth at 40°C	v	-	-	-	-	-	-	+	+	+	-	-	+
Tyrosinase	-	-	v	-	-	v	-	+	-	-	-	-	-
Utilization of betain	+	+	+	+	+	+	v	+	+	+	-	+	+
Acid production from:													
Trehalose	v	v	+	+	-	-	-	+	+	-	-	+	+
Xylose	+	-	+	+	+	+	+	+	-	+	-	+	+
L - Arabinose	-	-	+	+	v	+	-	+	-	-	-	-	+
Mannose	+	+	+	+	+	+	+	+	-	+	-	+	+
L -Rhamnose	-	-	+	+	-	-	v	+	-	+	-	-	-
Acid production from:													
Sucrose	-	v	v	-	+	+	+	-	-	-	-	-	-
Cellobiose	-	-	+	-	-	v	-	+	-	-	-	-	+
Lactose	-	-	+	-	-	-	v	+	-	+	-	-	-
Raffinose	-	-	v	-	-	-	-	-	-	+	-	-	-
Salicin	-	-	+	-	-	-	-	-	-	+	-	-	+
Inuline	-	-	-	-	-	-	-	-	-	-	-	+	+
Inositol	-	v	+	-	+	+	+	+	-	-	-	+	+
Sorbitol	-	-	+	+	+	+	+	+	-	+	-	-	+

Table 2. Contd.

Character [b]	Cluster number[a]												
	A	**B**	**C**	**D**	**E**	**F**	**G**	**40**	**201**	**29**	**122**	**405**	**406**
Mannitol	-	+	+	+	+	+	+	+	-	+	-	+	+
Adonitol	-	v	+	-	v	-	-	-	-	-	-	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	+
L -Arabitol	-	+	+	+	+	+	+	+	-	-	-	+	+
Maltose	-	-	v	v	-	+	v	+	-	-	-	-	-
Etanol	v	-	v	+	-	+	v	+	-	+	-	+	+
Melibiose	v	v	+	+	-	+	+	+	+	+	-	+	+
Erythitol	v	-	-	-	-	-	-	-	-	+	-	-	-
N - Propanol	v	-	v	+	-	+	v	+	-	+	-	-	+
Geraniol	v	-	-	-	-	-	-	-	-	-	-	-	-
Utilization for growth:													
Acetate	+	+	+	+	+	v	+	+	+	+	+	+	+
Malonate	+	v	+	+	+	+	+	+	-	+	-	+	+
maleate	+	+	+	+	+	+	+	+	+	+	-	+	-
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+
D-tartrate	-	-	-	-	+	-	-	-	-	+	-	-	-
L-tartrate	v	v	-	-	-	-	-	-	-	-	-	-	+
Benzoate	v	-	-	-	-	-	-	-	-	-	-	+	-
Arginine	+	+	+	+	v	+	v	+	+	-	-	-	+
β-Alanine	+	+	+	+	-	-	-	+	+	+	-	+	+
Quinat	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxalate	v	v	-	-	-	-	-	-	+	-	-	+	+

[a],Cluster number from numerical analysis of phenotypic data; +, 80% or more strains positive; v, between 21 - 79% strains positive (numbers in parentheses are percentages of strains that tested positive); -, 80% or more strains negative.

500 mMKCl, Tris-Hcl (PH = 8.4), 200 mM Amoniom sulfate) 20pmol of the two opposing primers (Cinna Gen of Iran), 200 μM of each dexoynucleoside triphosphate (Cinna Gen of Iran), 1.5 mM $MgCl_2$ (Cinna Gen of Iran), 1 μl (/ ng) of template DNA and 2.5 U of *Taq* DNA polymerase (Cinna Gen of Iran). Amplification were performed with a DNA thermocycler (Eppendorf AG, Germany) with an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min (Widmer et al., 1992; Porteous et al., 1985).

Data analysis

The clustering of the strains was performed by the unweighted pair group method using arithmetic average (UPGMA) and simple matching coefficient using MVSP 32 software.

RESULTS

The collected samples showed a wide range of sheath and grain symptoms. Pathogenic strains were often isolated from samples with symptoms that include longitudinal brown to reddish brown necrosis 5 mm wide extending the entire length of flag leaf sheath. Panicles emerging from these samples often were affected so that 1/3 to 1/4 of panicles failed to emerge from the boot. These panicles produced discolored brown to reddish brown and poorly filled grain. These symptoms mostly were observed on samples from high lands (Savadkouh and Kiasar). These symptoms were distinct from sheath and seed discoloration on the samples from other regions. Sheath discoloration symptoms in coastal low lands and the plains especially in Mahmoodabad where no pathogenic strain was isolated, were different from above-mentioned typical symptoms. The latter samples showed a dark brown to black sheath discoloration which mostly extended to stem.

Over 800 bacterial strains were isolated from 207 samples collected from 12 cities (Figure 1) and tested for HR and/or pathogencity on rice plant (Table 1). Distribution and isolation frequency of the 64 strains, which were grouped into clusters (Figure 2) are presented in Table 3. No pathogenic strain was isolated from eight samples collected from Ramsar. A wide range of differences in morphology and color of colonies were observed on isolation plates. Yellow and fluorescent colonies, which were non-pathogenic (with negative HR), were predominant. These colonies grow very rapidly and

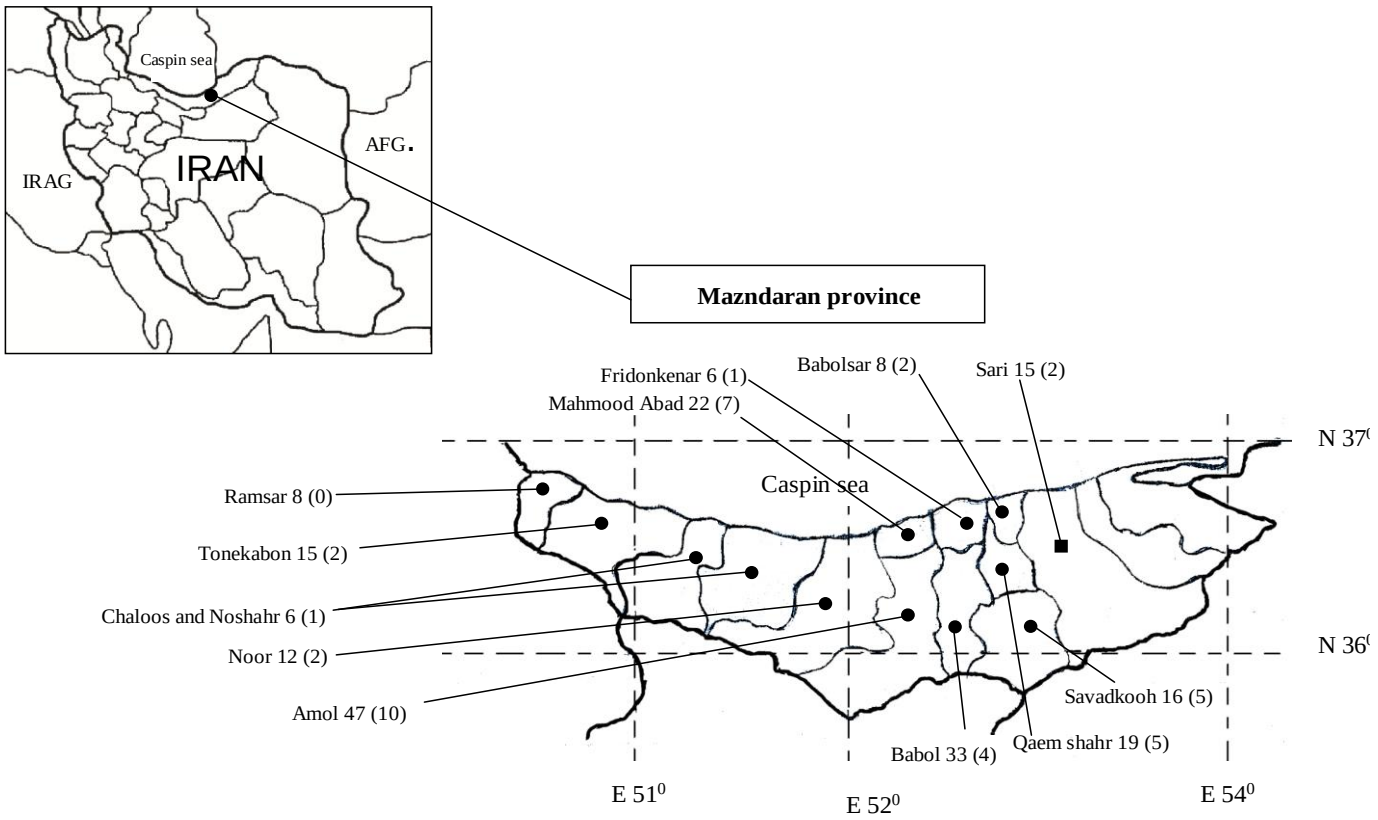

Figure 1. Sampling sites and respective number of Healthy and infectedsamples collected from the major growing region of the Mazandaran province over a period of 4 years (2002 - 2005).

interfere with the isolation procedure. Pathogenic and/or fluorescent HR positive strains were isolated from samples with typical symptoms of bacterial sheath rot and these colonies were predominant on the plate. These strains showed HR in test plant less than 12h and considering higher growth rate, colony morphology, rapid and intensive fluorescent pigment production were different from saprophytic fluorescent bacteria. These isolates were recovered from both seeds and sheaths.

Pathogenicity test

All pathogenic strains on seedling after 2 - 3 days of inoculation showed, elongated water soaked blotches on the leaf sheaths. These blotches developed into a brownish black necrosis that eventually spread the full length of the sheaths. Plants inoculated at booting stage showed progressive water - soaking in the inoculated region after 2 - 4 days. The lesions became necrotic and progressed up and down the inoculated region, induced the necrotic stripe on the sheath that eventually spread the full length of the sheaths. Panicles emerged from these plants were poor and produced brown to reddish brown grains that often were sterile. In some cases, the symptoms were restricted to the inoculation point as gray to dark brown spots. These strains and those which caused only discoloration on seeds were considered as weak pathogen. For most of the strains, the symptom severity was less in greenhouse than in the field. Although the observed symptoms were generally the same, the severity of the produced lesions greatly varied among the strains.

Biochemical characteristics

The tested strains were divided into two groups according to fluorescent pigmentation on king's B medium. The non-fluorescent group only contained strain 29 while the other strains belonged to the fluorescent group. The 70 tested strains were grouped into seven distinct clusters and six groups with one member based on the biochemical characteristics (Table 2, Figure 2). Strains of cluster A and strains 40 and 122 were not pathogenic on rice. Cluster A contained seven strains with characteristics similar to *Pseudomonas putida.* Cluster B included strains that in 84% similarity were grouped into the distinct B1, B2 and B3 sub-clusters. In B1 and B2 sub-clusters, only strain 14 caused grain discoloration and other strains were not pathogenic on rice. The strains in B2 sub-cluster were similar to *P. putida*al though they showed differences in gelatin hydrolysis, lecithinase and tobacco hypersensitivity. B2 sub-cluster (strains 104, 108

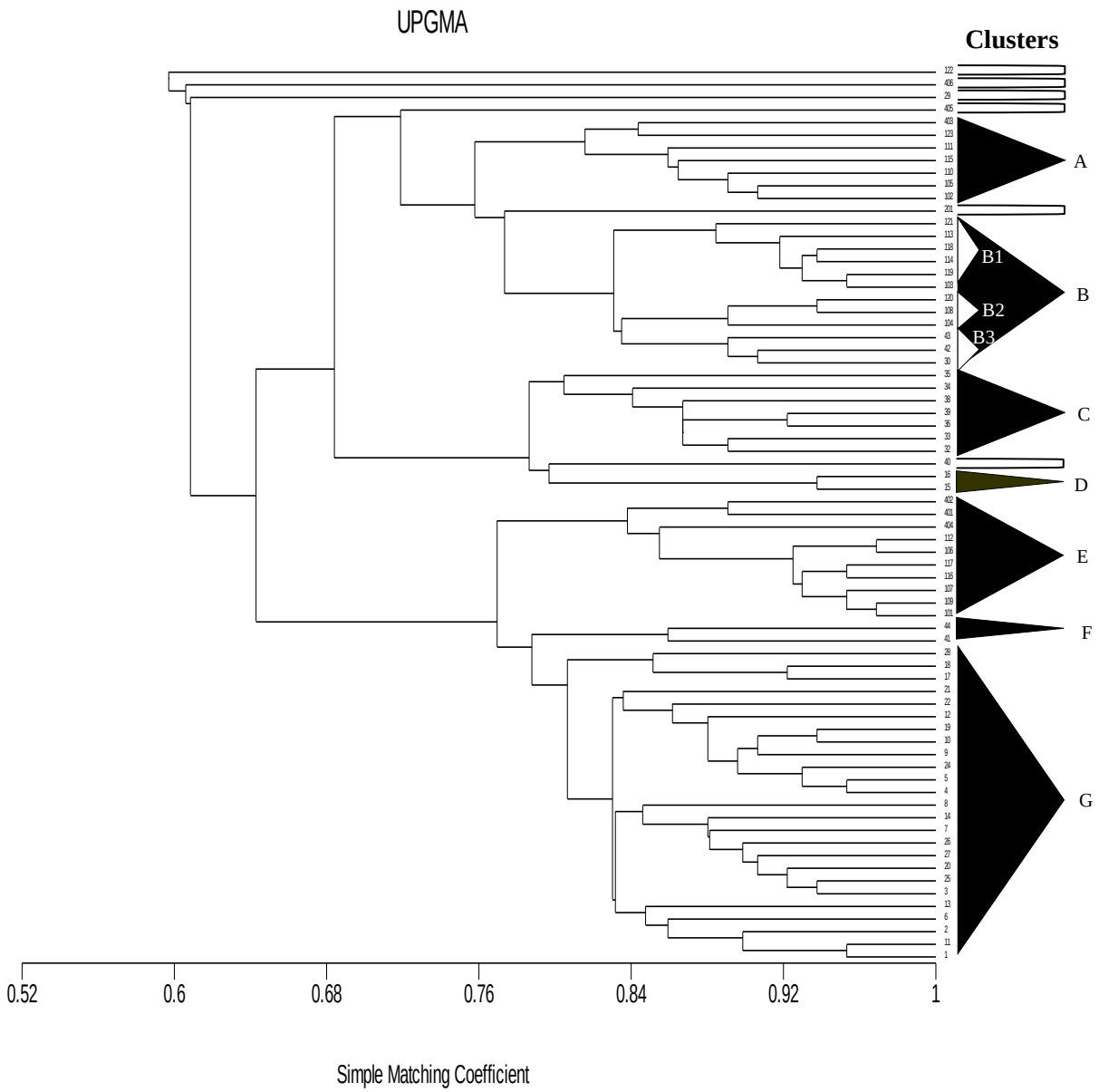

Figure 2. Cluster analysis of phenotypic data of bacterial strains isolated from rice samples with sheath rot and grain discoloration collected in Mazandaran province.

and 120) contained strains similar to *P. fluorescens* bv III. They differed from B1 cluster in acid production from trehalose, sucrose, inositol and adonitol. B3 sub-cluster contained the strains (30, 42 and 43) that caused disease on rice at both seedling and booting stages. These strains differentiated from all pathogenic fluorescent pseudomonades associated with sheath rot and grain discoloration. They were similar to *P. fuscovaginae* for soft rot of potato, 2-ketogluconate production and acid production from inositol and similar to *P. marginalis* for nitrate reduction, lecithinase and growth at 37°C. They were variable for acid production from sorbitol, trehalose, sucrose and adonitol. Cluster C included seven strains that all were pathogenic on rice seedling but only strains 32 and 34 caused disease at booting stage. Phenotypic characters of this cluster were variable which differentiated it from all common pathogenic fluorescent pseudomonads associated with the sheath rot (Garden et al., 2002; Schaad et al., 2001; Zeigler and Alvarez, 1990). Phenotypiccharacters of strain 35 which is grouped relatively distant from this cluster were similar to *P. marginalis.*

Cluster D contained two strains (15 and 16) that caused severe disease on rice in both stages. These strains had similar characteristics to *P. fuscovaginae* in comparison with other pathogenic *Pseudomonas*. When the eight

Table 3. Origin and isolation frequency of (pathogenic) strains associated with sheath rot complex and grain discoloration of rice in Mazandaran province (Iran).

Origin	Sample (total)[a]	Pathogens[b]	Cluster[c]						
			A	B	C	D	E	F	G
Amol	10 (47)	11	2	4	1	1	2	-	6
Babol	4 (33)	4	1	-	1	-	-	-	4
Babolsar	2 (8)	2	1	-	-	-	-	-	2
Chalos and Noshah	1 (6)	1	-	1	1	-	-	-	-
Fridonkenar	1 (6)	1	-	1	-	-	-	-	1
Ghaemshah	5(19)	5	-	-	1	-	-	-	5
Mahmodabad	7 (22)	7	-	2	2	-	-	2	4
Noor	2 (12)	2	-	-	-	-	-	-	1
Ramsar	0 (8)	0	-	-	-	-	-	-	-
Sari	2 (15)	2	-	2	-	-	2	-	-
Savadkoh	5 (16)	5	-	-	-	-	3	-	3
Tnokabon	3 (15)	3	1	2	-	1	-	-	-

a, Number of samples from which the pathogenic strains were isolated and (total sample); b, number of pathogenic strains; c, number of strains grouped according to numerical analysis of phenotypic data (one member groups are not listed 7 in Table).

biochemical tests useful in identifying *P. fuscovaginae* (Rott et al., 1991) were used for comparison, these strains differed from *P. fuscovaginae* for production of 2-ketogluconate and acid from sorbitol. Moreover, comparison with more phenotypic characteristics (55 tests) showed they were differentiated from *P. fuscovaginae* (Miyajimaet al., 1983). These strains differed from *P. fuscovaginae* for urease test, production of H_2S from thiosulfate, growth at 3% NaCl, lecithinase, acid production from xylose, melibiose and L-arabitol. They were variable for growth at 37°C, acid production from maltose and N-propanol.Strain 40 had similar characteristicsto*P. Fluorescens* bv. I. This strain was not pathogenic on rice. Strain 201 which was isolated from seedling with blight had similar characteristics to *P. fuscovaginae*. In comparison to eight biochemical tests (Rottet al., 1991), only this strain had complete similarity to *P. fuscovaginae*al though more phenotypic characters differentiated it from *P. fuscovaginae*.

Reference strains 405 (*B. andropogonis*) and 406 (*B. gladioli pv. gladioli*) were grouped into separate clusters with one member. Strain 29 was pathogenic on rice in both seedling and booting stages. This strain was phenotypically similar to *A. avenae* subsp. *avenae.* Strain 29 produced the brown stripe on the leaf midrib of inoculated seedling and was the only pathogen recognizable by a typical symptoms expression in the pathogenicity tests on seedling. It was phenotypically distinct from fluorescent strains as well and grouped into different one member group. Its phenotypic characteristics were similar to those reported by Schaad et al. (2001) for *A. avenae* subsp. *avenae* except for utilization ofbetain, growth on arabinose and starch hydrolysis (Table 2).

Cluster analysis of phenotypic data grouped all oxidase and arginine dehydrolase negative strains except for strain 122 into E, F and G clusters. According to their phenotypic characteristics, all of these strains were identified as *P. syringae* (Table 4). The E cluster contained seven rice strains and the standard strains *P. s.* pv*. Atrofaciens P. s. pv. panici* and *P. viridiflava* at 80% similarity. All rice strain of E cluster showed weak to strong pathogenicity on rice at booting stage. Only strain 109 was pathogenic on rice seedling (Table 1). All strains of this cluster showed weak levan production in comparison with *P. s. pv. atrofaciens* standard strain. These strains were distinct from F and G clusters in levan production, alkaline production from D-tartrat. F cluster contained only two (41 and 44) strains of which only strain 44 was pathogenic on rice sheath. Phenotypic characteristics of this cluster showed its similarity to *P.syringae*.

However, some characteristics such as growth at 37°C, cellobiose utilization and 2-ketogluconate production differed from *P. syringae* and strains in E and G clusters. ClusterG contained 25 strains with characteristics similarto *P. syringae*, however, they were variable in some important tests including, arbutin hydrolysis and betain. Also, they were variable in 2-ketogluconate production, production of reducing substances from sucrose, H_2S production from cysteine and thiosulfate, lecithinase, tyrosinase, acid production from maltose, rhamnose, lactose, salicin, ethanol, n - propanol, and alkalines production from arginine.

Genus-specific primers

Ps-for/Ps-rev primers amplified a DNA fragment of 990 kb in all strains except for strain 29. This band was not amplified in negative controls including *B. andropogonis*

Table 4. Comparison of the phenotypic characteristics of *P. syringae* strains associated with sheath rot and grain discoloration of rice in Iran (Mazandaran province) and published characters of *P. syringae* described by Schaad et al. (2001).

Character	*P. syringae*[c]	Cluster number [a]		
		E (7[b])	F (2)	G (25)
Fluorescent pigment	+	+	+	+
Levan production	D	V (5) b	-	- (5)
Oxidase	-	-	-	-
Arginine dehydrolase	-	-	-	- (1)
Soft rot of potato	-	-	-	-
Tobacco hypersensitivity	+	+	+	+
Growth at 37°C	-	-	-	- (2)
Nitrate reduction	-	-	-	-
Gelatin liquefaction	D	V (5)	-	+ (20)
Utilization for growth:				
Mannitol	D	+	+	+
Geraniol	-	-	-	-
Sorbitol	+	+	+	+
Cellobiose	-	-	V (1)	-
benzoat	-	-	-	-
Sucrose	+	+	+	+
D -Tartrat	- (D)	+	-	- (4)
L -Tartrat	- (D)	-	-	-
Trehalose	-	-	-	-
Ice nucleation	+	+	+	+
L -Rhamnos	-	-	-	V (15)

[a],Cluster number from numerical analysis of phenotypic data; [b],number of strains grouped according to numerical analysis of phenotypic data; [c], published characters of *P. syringae* described by Schaad et al. (2001); [D], some pathovars are positive; +, 80% or more strains positive; V, between 21 - 79% strains positive (numbers in parentheses are percentages of strains that tested positive); -, 80% or more strains negative.

and *B. gladioli* pv. *gladioli* (Figure 3). Accordingly all strains except the non-fluorescent pathogenic strain 29 which was confirmed to be *Acidovorax*, approved to be Pseudomonas.

DISCUSSION

Eight hundred bacterial stains were collected from rice plants with the sheath rot complex and grain discoloration, of which only 5.3% (43 strains) were pathogenic and majority of this bacteria were saprophytic. The earlier studies (Miyajima et al., 1983; Cottyn et al., 1996a) also reported that less than 15% of their collected strains were pathogenic. These pathogenic strains were isolated from 42 out of 207 collected samples (20.28% of samples) which confirmed the importance of bacterial agents in sheath rot complex and grain discoloration as previously reported by other researchers (Cottyn et al., 1996a, b; Adorada et al., 2013).

In pathogenicity tests, the pathogenic strains showed similar symptoms, lacked any differential symptoms for etiological purpose. Nevertheless, the disease severity was varied among the strains and they were mainly less severe in greenhouse experiments.The large proportion of samples which showed disease symptoms may be caused by abiotic agents or isolation problem.Based on phenotypic characteristics and specific primers, our strains belong to the genera including *Acidovorax* and *Pseudomonas*. The only strain of *Acidovorax* (Strain 29) identified as *A. avenae* subsp. *Avenae* which is seed born and has been detected in seed lots from many countries (Cottyn et al., 1996a; Webstar and Gunnell, 1992) and identified as a common agent associated with sheath rot complex disease (Cottyn et al., 1996a,b; Zeigler and Alvarez, 1990). Strain 29 was isolated from seeds showing symptoms and the role of this strain in sheath rot complex disease remains unclear.

Based on polyphase taxonomic studies, *Pseudomonas* have been revealed to comprise rRNA group I species, it includes the strain *P. aruginosae* and other fluorescent Pseudomonads such as *P.fluorescens*, *P. putida*, *P. syringe* and *P. fuscovaginae* (Anzaiet al., 2000; Kersters et al., 1996; Porteous et al., 1985) which are the principal bacteria known to infect rice grain and sheaths (Zeigler et al., 1987; Cottyn et al., 1996a; Xie et al., 2012). Based on

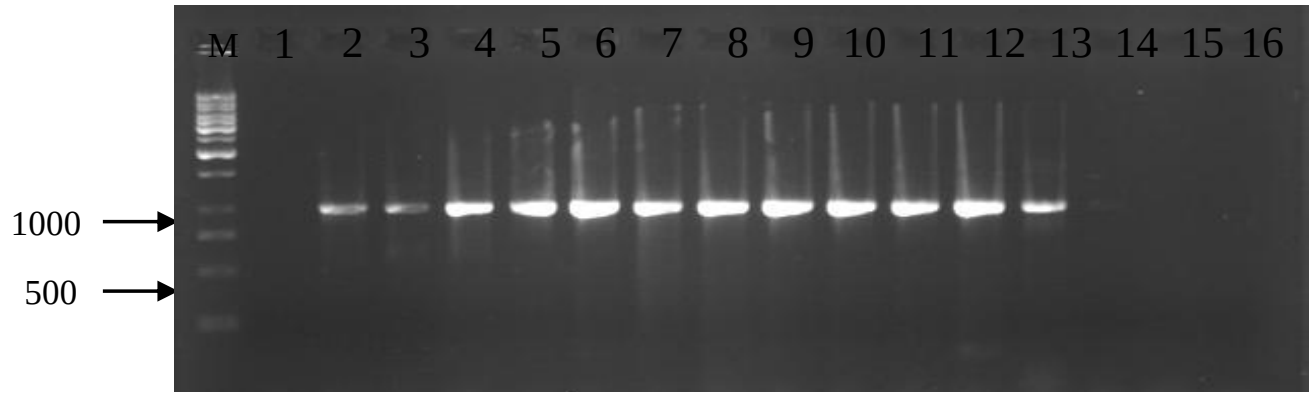

Figure 3. Agarose gel electrophoresis of *Pseudomonas* selective studied strains with *Pseudomonas* specific primers (PS - for , PS - rev) .M, Ladder 1 Kb; 1, negative; 2, *P.s.*pv. *syringe*; 3, *P. fuscovaginae*; 4, *P. marginalis*; 5, 101; 6, 102; 7, 103; 8, 104; 9, 105; 10, 106; 11, 107; 12, 108; 13, 109; 14, *B. andropogonios*; 15, *B. gladioli* pv. *gladioli*; 16, *Acidovoraxavenae*.

phenotypic characteristics, the tested strains belonging to this genus, could be clearly separated into two groups; those positive for arginine dihydrolase and oxidase and those negative for arginine dihydrolase and oxidase, identified as *P. syringae*. According to the resulting dendogram of phenotypic characteristics at 80% similarity level, strains of the first group (positive for arginine dihydrolase and oxidase) were grouped in clusters A to D and the single-member groups 201 and 40. Pathogenic strains from this group belong to *P. putida*(strain 114), *P. marginalis* (strain 32 and 34) and the unidentified species of *Pseudomonas* include strain 201, clusters D and subcluster B3, respectively. Strains of sub-cluster B3 display intermediate characteristics of *P. marginalis* and *P. fuscovaginae* which were different from those of known arginine dehydrolase-oxidase strains (Schaadet al., 2001; Zeigler and Alvarez, 1990; Cottynet al., 1996 a,b; Webster and Gunnell, 1992).Strains 32 and 34 have phenotypic characteristics similar to *P. marginalis*. Phenotypic characteristics of Strains of cluster D (strains 15,16) and strain 201 did not fit with any of described species of phytopathogenic flouresent Pseudomonas (Schaadet al., 2001; Zeigler and Alvarez, 1990; Cottynet al., 1996 a, b; Webster and Gunnell, 1992), however they show more similarity to *P. fuscovaginae* than the other pathogenic strains. However, regarding the difference in eight biochemical characteristics (Rottet al., 1991) and 55 phenotypic characteristics (Miyajimaet al., 1983) these strains are considered to be distinct from *P. fuscovaginae*.

Previous studies on samples from Brundi (Duveiller et al., 1990; Duveiller et al., 1998 b) Colombia (Zeigler and Alvarez, 1987) and Madagascar (Rott et al., 1989) have demonstrated strains with similar characteristics to these pathogenic strains. The determination of the exact taxonomic position of these strains needs complementary studies especially investigations at molecular level.

The second group of *Pseudomonas* strains includes the strains which were negative for arginine dehydrolase-oxidase. As the major bacterial agents associated with sheath rot complex disease in Iran, these strains are grouped in E, F and G clusters. Analysis of phenotypic characteristics explicitly revealed that these strains belong to *P. syringae*, which has been also associated with sheath rot complex disease in Hungary (Ou, 1985), Australia, Chile and several Asian countries (Ou, 1985; Cottynet al., 1996a; Rottet al., 1989; Webstar and Gunnell, 1992). E, F, and G clusters differed from each other for characteristics which are not taxonomically important. Besides, there was no difference in their geographical origin or tissue of isolation.These clusters were considered as taxonomically similar to *P. syringae*. Since phenotypic characteristics are not sufficient for clustering the strains at infraspecific level (pathovar), (Manceau and Horvais, 1997; Cottyn et al., 1996; Joana et al, 2007) distinction of strains through comparison with the common rice disease associated *P. syringae* pathovars was not possible. Taxonomic identification of these strains at subspecies level (pathovar) requires complementary molecular studies such as comparison of electroforetic patterns of total protein, analysis of ITS regions (Manceau and Horvais, 1997; Olczak-Woltman et al., 1983) and DNA fingerprinting (Jaunetet al., 1995; Little et al., 1996; Louwset al., 1994), which are now in progress.

The fluorescent non-pathogenic species such as *P. putida* and *P. fluorescens* were commonly recovered from the samples with disease symptoms and determination of their possible role in development of disease syndrome needs further study. Strains similar to *P.s.* pv. *panici* (Elliott) Young et al. 1987, causing bacterial brown stripe of rice seedling, *B. glumae* causing grain rot (Mew and Cottyn, 1997; Ou, 1985), panicule blight (Yuan et al., 2004) and as common bacteria associated with sheath rot complex (Zeigler and Alvarez, 1990; Cottyni et al., 1996), *B. gladioli* causing leaf - sheath browning of rice (Hiroyuki et al., 2006) were not detected in the collected samples.

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